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Intrinsic and Chemically Produced Microheterogeneity of *Staphylococcus aureus* Enterotoxin Type C

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Staphylococcus aureus enterotoxins C_1 (SEC_1) and C_2 (SEC_2) produced from 50-liter quantities of crude culture supernatants were purified chromatographically in a neutral or acid milieu. Microheterogenity of SEC was markedly increased by treatment of the purified toxin with alkali, and new, more acidic charged species appeared. SEC_2 was more heterogenous than any of the other *S. aureus* enterotoxins and was affected only slightly by treatment with alkali. Prolonged incubation of the organism during production of the SEC_2 produced changes in charged species that may be related to a bacterial deamidase, since similar changes were not seen with alkaline treatment of the purified toxin. Although SEC_1 and SEC_2 showed complete identity immunologically, they are separate, distinct toxins, and alkali treatment of SEC_1 did not produce SEC_2.

The heterogeneity of *Staphylococcus aureus* enterotoxins was first described by Baird-Parker and Joseph (2). Confirmation of this heterogeneity was reported by Schantz et al. (11) for *S. aureus* enterotoxin B (SEB) by means of electrophoresis on starch gel. By using isoelectric focusing, Metzger et al. (8) demonstrated that SEB consisted of four species. The two major components were stable at neutral pH at 4 C. Spero et al. (13) studied the effects of an alkaline milieu on SEB and found that there was progressive amide hydrolysis at 37 C leading to a loss in the alkaline components with concomitant development of the more acidic ones. The several species formed were one charge apart.

In the original description of the isolation of *S. aureus* enterotoxin C_1 (SEC_1), Borja and Bergdoll (3) found two components by starch gel electrophoresis that were attributed to either buffer interaction or dimerization. Extreme heterogeneity was observed with a preparation of *S. aureus* enterotoxin C_2 (SEC_2) obtained by purification from a culture that had been incubated for 72 h at 37 C (6). *S. aureus* enterotoxin production, however, has been reported to be complete by 10 to 18 h (7, 9). The toxin was thus exposed unnecessarily for a long period to an alkaline milieu and possible bacterial deamidases before purification. Furthermore, some methods of purification of SEC_1 (3) and SEC_2 (1) utilize a period of initial concentration of the alkaline culture filtrate that could affect qualitatively and quantitatively the homogeneity of the toxins before purification.

In this report, we describe methods for purification that promptly remove the toxin to a neutral or acid pH. The effects of alkali at 37 C on SEC_1 and SEC_2 purified in this manner are compared with untreated purified toxins. In addition, SEC_2 purified from a 72-h fermentation was studied for possible differences in isoelectric composition.

MATERIALS AND METHODS

S. aureus strains. Strain 137-H-2 was utilized for production of SEC_1; strain 361 was utilized for production of SEC_2. All cultures were maintained in lyophilized form, and a new ampoule was used for each experiment.

Fermentation. All studies used a 70-liter fermentor (Fermentation Design, Allentown, Pa.). Controlled settings consisted of 400-rpm agitation, 10 liters/min of air sparge, and 37 C temperature. All fermentations were carried out for 18 h except where noted.

Medium. All fermentations were carried out in 50 liters of medium containing 4% NAK (Sheffield Chemical Co., Norwich, N.Y.), 1% yeast extract (Difco, Detroit, Mich.), and 0.2% glucose (wt/vol).

Centrifugation. After fermentation the culture was centrifuged at 16,000 rpm by using a continuous-flow head (Lourdes, Old Bethpage, N.Y.).

Demineralization. All crude bacterial supernatants were partially desalted by passing through a demineralizer cartridge (Barnstead, Boston, Mass.).

Chromatography. CG-50 (Mallinckrodt, Millville, N.J.) was activated by alkaline and acid treatment. After activation, the resin was equilibrated at the appropriate pH with phosphate buffer. The washed resin was stirred into the diluted culture supernatant. The resin was allowed to settle and was then poured into a chromatography column. Carboxymethylcellulose (CM-cellulose) (Bio-Rad, Richmond, Calif.)

was equilibrated with phosphate buffer at the appropriate pH and then washed with distilled water. The CM-cellulose was stirred into the diluted crude toxin, allowed to settle, and then poured into a chromatography column. Elution characteristics are given under each purification scheme.

Isoelectric focusing. Isoelectric focusing in sucrose gradients was carried out as recommended by LKB (Stockholm, Sweden). Isoelectric focusing in gels containing pH 3 to 10 ampholines was done at 17 C according to the method of Wrigley (14).

Antiserum. Anti-SEC_1 was prepared by repeated intramuscular injections of the major component of purified SEC_1 isolated by electrofocusing. The preparation was mixed with complete Freund adjuvant (Difco) for injection into goats.

Ouchterlony (10) double diffusion. One percent Ionagar (15 ml) dissolved in pH 8.3 borate buffer was layered onto glass plates (8 by 10 cm). Three-millimeter holes were punched at 5-mm intervals in a circular pattern with a well in the center; 5 μl of antigen or antisera was placed in each well.

Purification of SEC_1. The culture supernatant from strain 137-H-2 was diluted 1:5 with distilled water and the pH was adjusted to 6.2 with phosphoric acid. CG-50 (350 g) equilibrated at pH 6.2 with 0.01 M phosphate was added to the diluted culture supernatant. The toxin was eluted from the column with 0.5 M phosphate buffer containing 0.25 M NaCl (pH 6.8). The toxin peak was dialyzed at 4 C against distilled water to reduce the salt concentration. The dialyzed toxin was diluted 1:10 with distilled water; 100 g of CG-50 equilibrated at pH 6.8 was added. The column was washed with distilled water and the toxin was eluted with 0.15 M Na_2HPO_4. The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 6.2); 200 g of CM-cellulose equilibrated at pH 6.2 was added to the dialyzed toxin. The toxin was eluted from the column by a linear gradient (0.02 to 0.07 M phosphate buffer, pH 6.2 to 6.8). The toxin-containing fractions were combined, dialyzed against 0.01 M phosphate buffer (pH 7.0), and lyophilized.

Purification of SEC_2. The supernatant from a culture of strain 361 was prepared as for SEC_1, except that the pH was adjusted to 5.6. CG-50 (350 g) was equilibrated to pH 5.6 with 0.005 M phosphate. Elution of toxin was accomplished with 0.5 M phosphate buffer plus 0.5 M NaCl at pH 6.2. The toxin-containing eluate was dialyzed first against distilled water and then against 0.01 M phosphate buffer at pH 6.0. CM-cellulose (200 g) equilibrated at 0.01 M, pH 6.0, was added to the dialyzed toxin. The toxin was eluted from the column by using a linear gradient (0.01 to 0.05 M phosphate buffer, pH 6.0 to 6.8). The toxin-containing fractions were combined and dialyzed against 0.03 M phosphate buffer, pH 5.7. Hydroxylapatite (150 g) was swollen in 0.03 M phosphate buffer at 5.7. A column (5 by 60 cm) was poured at 4 C, and the column was further equilibrated with buffer at 4 C. The toxin was slowly passed through the column and then washed with the equilibrating buffer. Elution was accomplished by using a linear gradient (0.2 to 0.4 M phosphate, pH 5.7). The toxin peak was dialyzed against 0.01 M phosphate buffer (pH 7.0) and then lyophilized.

SEC_2 (72 h) purification. The supernatant culture fluid of a 72-h fermentation of strain 361 was concentrated to 4 liters by membrane filtration (Amicon TC1D with UM-10 membrane; Amicon Corp., Lexington, Mass.) at 4 C. The concentrated supernatant was dialyzed against distilled water and then centrifuged to remove any precipitate. CM-cellulose (Microgranular no. 52, Whatman; Reeve-Angel, Clifton, N.J.) was equilibrated with 0.01 M phosphate buffer at pH 5.5. A column (2.5 by 50 cm) was poured at 4 C. A 500-ml aliquot of toxin previously dialyzed against 0.01 M phosphate buffer, pH 5.5, was run slowly through the column. The column was washed with the equilibrating buffer and the toxin was eluted with a linear gradient (0.01 to 0.07 M phosphate buffer, pH 5.5 to 6.8). The toxin-containing peak was dialyzed against 0.01 M phosphate-buffered saline, pH 7.0. The toxin was further purified by molecular sieving on a Sephadex G-75 column equilibrated with phosphate-buffered saline. A symmetrical toxin peak was eluted, dialyzed against 0.01 M phosphate buffer, pH 7.0, and lyophilized.

Alkaline treatment of purified toxins. Purified toxins were exposed to pH 9.0 at 37 C in 0.01 M tris(hydroxymethyl)aminomethane buffer (13).

RESULTS

SEC_1 purified by the described chromatographic method contained three components by electrofocusing (Fig. 1) in a sucrose gradient analytical instrument. The two major components had isoionic points of 9.19 and 8.83, which were determined at 4 C. Three bands were also demonstrated by gel electrofocusing (Fig. 2A). The most alkaline species by both procedures was present in only trace amounts, whereas the two major components were in a ratio of approx-

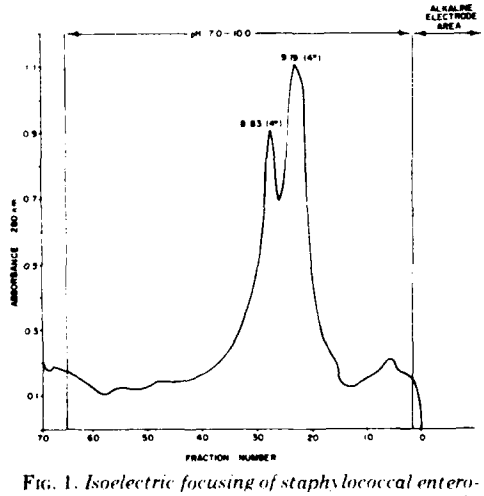

FIG. 1. *Isoelectric focusing of staphylococcal enterotoxin C_1, using pH 7 to 10 ampholine-sucrose gradient. Electrofocusing was performed at 4 C. The pH values were determined at 4 C.*

FIG. 2. *Isoelectric focusing of enterotoxin preparations in polyacrylamide gel, using pH 3 to 10 ampholines. (A) SEC_1; (B) SEC_1 treated with alkali; (C) SEC_2; (D) SEC_2 treated with alkali, (E) SEC_2 (72 h).*

imately 4:1, the pH 9.19 species being the major component.

Exposure of SEC_1 to pH 9.0 at 37 C for 10 days caused a marked increase in microheterogeneity and an almost complete loss of the two most alkaline species (Fig. 2B). There was also development of new, more acidic forms. The change in pattern was strikingly more drastic than that observed with SEB and indicated a much greater lability of SEC_1 to chemically induced deamidation. Significant alterations were even observed after dialysis against pH 9 buffer in the cold.

When purified SEC_2 was analyzed by a sucrose gradient analytical electrofocusing instrument, two sharp peaks with isoionic points of 8.40 and 7.10 were demonstrated (Fig. 3). In addition, there were at least three other components. Five major bands were seen by isoelectric focusing in gels (Fig. 2C). It is likely that the 8.40 component corresponds to the alkaline doublet of the gels and the broad shoulder on this peak is the major, next more acidic species seen in the gel (estimated pI = 8.1). The former components were greatly diminished after exposure of the purified toxin to pH 9.0 at 37 C for 10 days (Fig. 2D).

SEC_2 (72 h) had two major components with only trace amounts in the more alkaline region of the gels. The densitometric scan illustrated how markedly the relative concentrations of the several components were altered. In addition, a definite new acidic species was demonstrable (Fig. 2E and 4).

All toxin preparations showed lines of complete identity when examined by the Ouchterlony technique using anti-SEC_1 antiserum. Composite immunoelectrophoresis (Fig. 5) of the five preparations reveals that SEC_1 treated with alkali had decreased cathodic movement

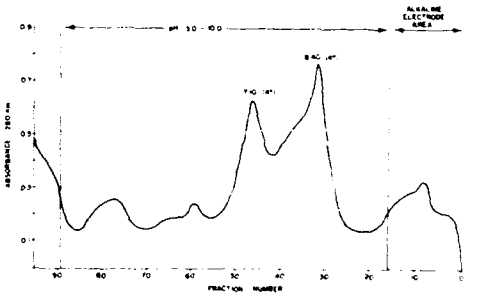

FIG. 3. *Isoelectric focusing of staphylococcal enterotoxin C_2, using pH 3 to 10 ampholine-sucrose gradient. Electrofocusing was performed at 4 C. The pH values were determined at 4 C.*

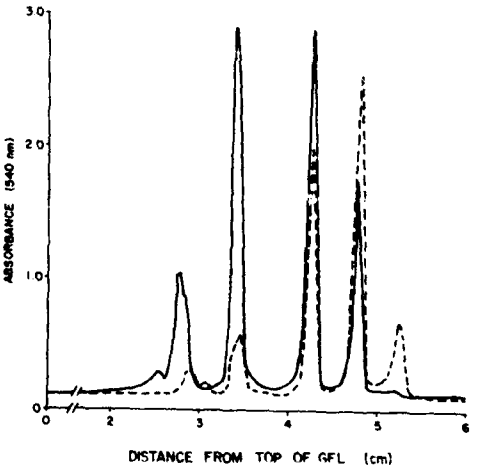

FIG. 4. *Densitometric scan of electrofocused gels. SEC_2 (——) and SEC_2 (72 h) (– –).*

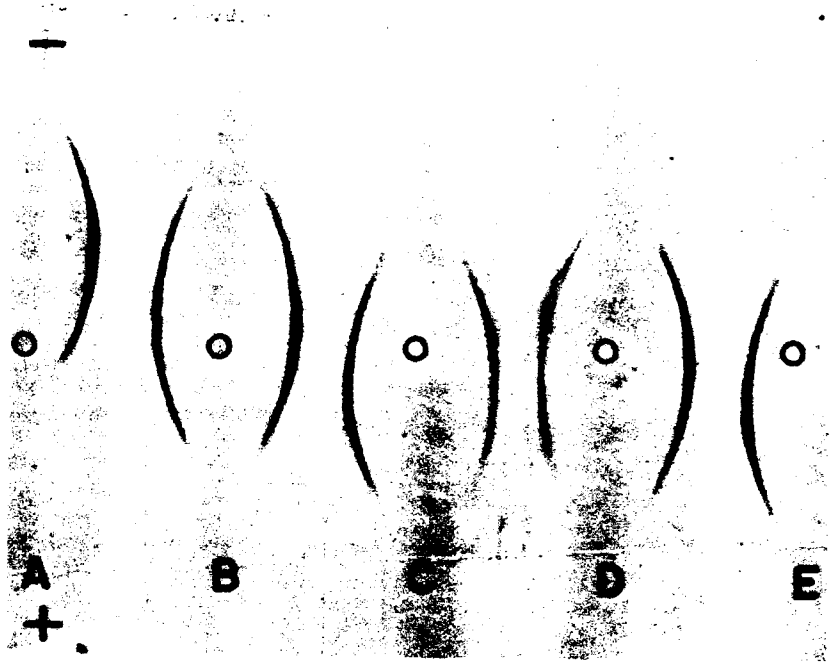

FIG. 5. *Immunoelectrophoresis of staphylococcal enterotoxins. (A) SEC_1; (B) SEC_1 treated with alkali; (C) SEC_2; (D) SEC_2 treated with alkali; (E) SEC_2 (72 h). Antiserum to SEC_1 was used in all troughs.*

compared with untreated SEC_1. Alkaline treatment of SEC_2 did not appear to change the average charge. The more acidic nature of SEC_2 (72 h) was demonstrated by its slight anodic movement.

Sodium dodecyl sulfate-acrylamide electrophoresis revealed that all C-type enterotoxin preparations co-migrated with purified SEB; therefore, the molecular weight is between 28,000 and 29,000.

DISCUSSION

SEC_1 consists of three components and has comparable microheterogeneity to *S. aureus* enterotoxins A (12) and B (8). In addition, the two major components of SEC_1 have isoionic points approximately 0.4 pH units apart, similar to the difference in isoionic points seen with the major components of *S. aureus* enterotoxins A (12) and B (8) and consistent with a single charge difference between isoelectric species. The behavior of SEC_1 treated with alkali is similar to that reported for SEB (13), i.e., a sequential conversion from more to less alkaline forms and the appearance of new, more acidic species. It is noteworthy, however, that SEC_1 is considerably more altered than SEB.

SEC_2 demonstrates more isoelectric pauci-dispersity than the other staphylococcal enterotoxins. Surprisingly, however, it is the most resistant variety to chemical deamidation induced by exposure to pH 9.0 and 37 C. Only the most alkaline species appeared to be affected, and the average charge, as evidenced by immunoelectrophoresis, was unchanged. A much greater change was brought about by prolonging the incubation of the SEC_2 culture to 72 h before isolation. The shorter time period suggests strongly that the change was produced enzymatically, presumably by a deamidase.

Two preparations of SEC_2 have been examined isoelectically by Dickie and co-workers (5, 6). Both were isolated after 72 h of incubation and by a procedure involving a preliminary concentration by dialysis against polyethylene glycol. In one instance the component present in highest concentration had a pI of 7.35 and in the other a pI of 6.50 (Their pI values were obtained by measurement of pH of the samples at 25 C. The values cited here were corrected to 4 C, our temperature of measurement, by the van't Hoff equation, assuming that the amino groups of the ampholines have ΔH of 10,000 cal/mol.) The 7.35 component probably corresponds to our 7.1 component, and the composition of the preparation, lacking our 8.4 component, is comparable

to our SEC_2 (72 h) material. The other preparation was considerably more deamidated and contained as its most alkaline species a fraction with a pI of 7.25, again comparable to our 7.1 component. It was thus devoid of both the 8.1 and 8.4 isoelectric species found in our preparations. It is apparent that considerable care must be taken in the isolation of the enterotoxins if one is to avoid degrading the proteins, and it would be well to characterize individual preparations by their isoelectric focusing patterns. It was found that when the initial steps of the isolation were not carried out promptly, SEC preparations were badly nicked. The extent of nicking is readily determined by sodium dodecyl sulfate-polyacrylamide electrophoresis in the presence and absence of β-mercaptoethanol.

The data support the report of Avena and Bergdoll (1) that SEC_1 and SEC_2 are different enterotoxins with identical immunological reactions. The gel isoelectric focusing patterns obtained after prolonged exposure of SEC_1 at pH 9.0 bore little resemblance to those of SEC_2.

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